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IMMUNOGENICITY OF FREEZE-DRIED AND DEEP-FROZEN BONE ALLOGRAFTS

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INTRODUCTION

It was not until the early 1950s that attempts were made to define the antigenicity of bone allografts and the relationship of immunogenicity to preservation techniques. Parameters used to evaluate this antigenicity included histological characterization of the host's cellular response surrounding the graft and an estimate of new bone formation (1, 2, 3, 4), altered patterns of second-set skin graft rejection (3, 5, 6, 7), changes in the regional lymph nodes draining the site of graft placement (2, 8, 9), and serological tests to identify antibodies against bone extracts (2, 10). Recently, more sensitive tests have been applied including cytotoxicity assays for humoral (7, 11) and cell-mediated immunity (CMI) (11, 12). All of these studies, with the exception of the work of Langer (11), indicated that freeze-drying and to a lesser degree deep-freezing of bone allografts markedly reduced or abolished their ability to evoke an immune response in the graft recipient. This is discussed in more detail elsewhere (13, 14).

Bonfiglio (1), Kruez (4), and Lundgren (12) have provided the only studies involving grafts placed orthotopically, and only Burwell (6, 8) and Kossowska-Paul (9) have examined the immunological response to fresh cortical and cortico-cancellous bone allografts separately. There have been no previously reported studies that have compared the immunogenicity of preserved cortical and cortico-cancellous allografts using assays for both humoral and cell-mediated immunity. The present study was undertaken to determine whether cortical or cortico-cancellous allografts preserved by deep-freezing or freeze-drying and placed in an orthotopic site were immunogenic using a sensitive immunological assay for cellular and humoral immunity. The allograft materials and freeze-drying technique employed were chosen to parallel those currently used by the U.S. Navy Tissue Bank for its ongoing clinical protocols.

MATERIALS AND METHODS

Nine randomly bred female Dutch belted rabbits (Rowmar Rabbitry, Mt. Airy, Maryland USA), weighing approximately 2 kilograms, served as bone allograft donors. Peripheral blood mononuclear cells pooled from four additional female Dutch belted rabbits were used as targets in the microcytotoxicity

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assay. A total of 31 randomly bred New Zealand white female rabbits (Bowmar Rabbitry), averaging 4 kilograms at the time of surgery, were used as bone graft recipients or normal controls and as the source of effector cells and sera for the assay. Twenty-seven rabbits received fresh or preserved allografts, and four underwent sham procedures in which no graft was inserted.

Bone was obtained sterilely and prepared as cortical chips from the shafts of the long bones or as small pieces of iliac crest containing both cortical and cancellous components. All bones were washed vigorously by hand in saline to remove loosely adherent hematopoietic elements. These grafts as well as those commonly used in clinical practice represent a composite tissue which contains varying amounts of osteogenic cells, collagen, matrix, hematopoietic and lymphoid elements, fibrous and adipose tissues, endothelial cells and nerves. Graft material was then either deep-frozen in a liquid nitrogen freezer (-196 to -170°C) for at least 4 weeks, freeze-dried to less than 5 residual moisture, or used fresh. Grafts were inserted under Innovar-Vet anesthesia (McNeil Laboratories Inc., Ft. Washington, Pennsylvania USA) in a bed created in the iliac crest by removing its outer cortex. Six to ten grams of alloqeneic bone were used in each animal which, when corrected for body weight, is equivalent to or greater than the average amount of graft material dispensed by the U.S. Navy Tissue Bank to treat a benign space occupying lesion of bone. Sera and lymphocytes to be used as effectors of cytotoxicity in the assay were collected 1-4 weeks following insertion of the grafts.

The microcytotoxicity assay for humoral and cell-mediated immunity was modified from techniques described by Brunner (15) and Lightbody (16). Details of the ^{51}chromium-release assay have been described in detail elsewhere (17). Briefly, a pool of Ficoll-Hypaque separated peripheral mononuclear cells stimulated with PHA from four Dutch belted rabbits served as target cells. The assay for humoral antibody was accomplished by adding 0.1 ml of the ^{51}chromium labelled target cell suspension (1 x 10^6 cells) to each well of a microtiter plate followed by 0.05 ml of test sera. The plate was incubated at 37°C for 30 min and 0.05 ml of a 1:8 dilution of guinea pig complement added. The plate was shaken on a microplate shaker, incubated at 37°C for 1 hr, and centrifuged for 5 min at 600 x g and 4°C. One-tenth milliliter of the supernatant fluid was removed and the ^{51}chromium release measured. Determination of cell-mediated lymphocytotoxicity (CML) was performed with a mononuclear effector cell to PHA stimulated target cell ratio of 100:1. Both humoral and cell-mediated cytotoxicity assays were run in triplicate and the mean and standard errors determined. The percent ^{51}chromium release in each case was calculated using the following equation:

$$\text{chromium release} = \frac{\text{CPM experimental well--CPM spontaneous release}}{\text{CPM of freeze-thawed well--CPM spontaneous release}} \times 100$$

Values of 5 or less were considered negative and values greater than 10 release were considered positive. Intermediate values were considered to be equivocal or "plus-minus (+)."

RESULTS

The results from this study are presented in Tables I and II and are summarized in Table III. The highest percent ^{51}Cr-release values over the 4-week period are reported. Using the effector to target cell ratio of 100:1, all recipients of fresh cortical and cortico-cancellous allografts demonstrated positive CML as did all four recipients of deep-frozen cortico-cancellous bone

(Table I). Freeze-dried cortical bone failed to elicit the presence of detectable lymphocytotoxicity in any of six animals tested, whereas three of five recipients of freeze-dried cortico-cancellous bone, and two of five recipients of deep-frozen bone became sensitized.

TABLE I

CELL-MEDIATED LYMPHOCYTOTOXICITY[1]

Graft Preparation	Percent ^{51}Cr-release	
	Cortico-Cancellous Grafts	Cortical Grafts
Fresh	34, 68	16, 13, 36
Freeze-Dried	9, 0, 16	8, 5, 4, 4, 0, 0
Deep-Frozen	30, 26, 44, 37	0, 0, 7, 18, 17

[1] Animals were assayed between 1 to 4 weeks after grafting.

TABLE II

ANTIBODY AND COMPLEMENT MEDIATED CYTOTOXICITY[1]

Graft Preparation	Percent ^{51}Cr-release	
	Cortico-Cancellous Grafts	Cortical Grafts
Fresh	19, 84, 85	16, 20, 20
Freeze-Dried	0, 0, 0, 9, 5	0, 0, 0, 0, 0, 19
Deep-Frozen	7, 25, 13, 18, 52	0, 2, 13, 13, 13

[1] Animals were assayed between 1 to 4 weeks after grafting.

Antibody mediated cytotoxicity was found to correspond closely with the presence of cell-mediated immunity (Tables II and III), with the exception that none of the animals receiving freeze-dried cortico-cancellous allografts and three of five rabbits receiving deep-frozen cortical bone demonstrated significant levels of cytotoxic antibody during the time tested. None of the sham operated animals tested demonstrated humoral or cellular immunity in this assay. These results suggest that the combination of freeze-drying and cortical bone is the least antigenic and deep-frozen cortico-cancellous bone the most sensitizing of the preserved allografts examined at this time interval following graft placement.

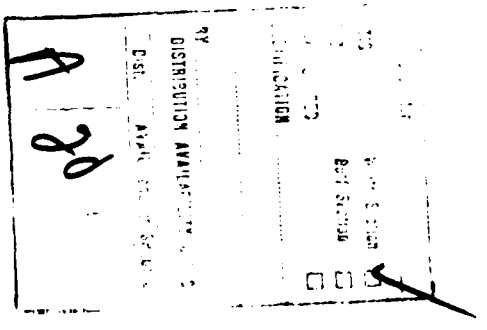

TABLE III

SUMMARY OF BONE ALLOGRAFT IMMUNOGENICITY

			Humoral Antibody			Lymphocytotoxicity[1]		
		N	+	+		+	+	
Fresh	Cortical	(3)	3[2]			3		
	Cancellous	(3)	3			2		
Freeze-Dried	Cortical	(6)	1		5		1	5
	Cancellous	(5)		2	3	3		2
Deep-Frozen	Cortical	(5)	3		2	2	1	2
	Cancellous	(5)	4	1		4		

[1] Chromium-release microcytotoxicity assay (see text).

[2] Number of individual rabbits giving designated response.

DISCUSSION

The RL-A locus of the rabbit, analogus to the HL-A locus of the human, controls a complex series of antigens which result in major histocompatibility differences between strains as well as pools of detectable antigens common to any one strain (18, 19). These tissue antigens are expressed on the surface of peripheral leukocytes (18) and fibroblasts (20); rodent bone and skin have been shown to share common antigens (3, 5, 6, 7). The microcytotoxicity assay used in this experiment depends upon the existence of major histocompatibility differences between the New Zealand white and the Dutch belted strains. Pools of Dutch belted rabbit leukocytes were used as target cells to maximize the number of antigenic differences within the major histocompatibility complex.

Previous attempts to demonstrate circulating antibodies induced by primary allografts of freeze-dried or deep-frozen bone have been unsuccessful (1, 7, 10, 11). Elves (7) has concluded that viable cells are needed to sensitize rats to bone allografts. Although in the present study both humoral and cell-mediated immunity was detected following allografts of deep-frozen cortico-cancellous bone, it is entirely possible that a small percentage of viable cells may have survived the deep-freezing process. It is unlikely, however, that viable cells remained in the freeze-dried preparations, which induced cell-mediated immunity in three of five recipients of cancellous grafts and only one detectable humoral response following implants of cortical materials.

Langer and his co-workers (11) studied fresh and frozen cortico-cancellous allografts placed heterotopically in rats. Cytotoxic humoral antibodies could be demonstrated following implantation of fresh but not frozen allografts. Leukocyte migration inhibition, an indicator of cell-mediated immunity, revealed that sensitization occurred in recipients of fresh and frozen allografts. The results reported here clearly demonstrate significant levels of both humoral and cell-mediated immunity following primary orthotopic allografts of deep-frozen cortico-cancellous bone.

The increased sensitivity of the microcytotoxicity assay used in the present study may reflect the sensitivity of the chromium release assay, and/or the fact that PHA stimulated lymphocytes are more sensitive to cytotoxic effects in in vitro assays (17, 21). It is also possible that the orthotopic placement of the allograft may have resulted in a greater likelihood of sensitizing the recipients. Although all the recipients of deep-frozen cortico-cancellous grafts produced both humoral and cell-mediated immunity and three of five recipients of freeze-dried cortico-cancellous bone grafts produced cell-mediated immunity, the same microcytotoxicity assay failed to detect significant sensitization to freeze-dried cortical grafts during the period of this study. The present study supports the contention that cortical bone is less immunogenic then cancellous bone, and a possible explanation must take into account the relatively greater cellularity (osteogenic and hematopoietic) of cancellous bone.

Freeze-drying has been shown to selectively alter certain receptors on the cell surface and to disrupt their functional capacity (22). Thus, although freeze-dried spleen cells were shown to be able to bind antisera, they were unable to bind or respond to plant mitogens. Differences have been reported in the antigenic requirements for the introduction of either a primary or secondary in vitro cytotoxic allograft response (23). In order to trigger a good primary response in vitro (mixed lymphocyte reaction) with allogeneic lymphoid cells, the LD (lymphocyte defined) gene product must be displayed in a functionally active form. However, in the induction of an efficient secondary response (CML), UV light irradiated lymphoid cells or membrane fragments were shown to be strongly immunogenic. Similar results have been reported using allogeneic freeze-dried membrane fragments (24). Such freeze-dried material induced poor proliferative and cytotoxic responses in normal spleen responder cells; however, re-exposure of immune responder T cells to allogeneic membrane fragments triggered the generation of highly reactive cytotoxic T lymphocytes. It would therefore appear that in certain instances the freeze-drying process alters the antigens involved in the primary response whereas the serologically defined (SD) antigens remain intact for the triggering of a secondary response. The data reported here varies slightly with these reports in that three of five animals which received freeze-dried cortico-cancellous bone did produce cell-mediated immunity; none of these animals, however, produced detectable antibody. Similarly, one of six animals receiving freeze-dried bone demonstrated a humoral response, and none had detectable levels of CML. These discrepancies could be explained on the basis of differences between the in vivo and in vitro models. In addition, it must be noted that the freeze-dried cortical bone did not induce cell-mediated immunity and therefore the differences seen could be due to quantitative amounts of immunogenic material. Further experiments need to be carried out in this in vivo model to determine whether or not freeze-dried cortical grafts are capable of initiating a secondary response in primed recipients. It will also be important to examine recipients of preserved bone allografts at other time intervals than presented in this preliminary study.

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RESUME

L'etude de l'immunogénicité des allogreffes osseuses conservées présente un intérêt croissant du fait de l'utilisation clinique de ces allogreffes. Dans le présent travail, un test de cytotoxicité avec mesure de relâchement du chrome a été utilisé dans le but d'étudier l'immunité cellulaire et/ou humorale de lapins recevant des allogreffes osseuses lyophilisées et congelées (-170° C) (corticale ou medullaire). Une allogreffe est placée en position orthotopique sur la crête iliaque d'un lapin. Les lymphocytes du donneur stimulés par la phytohémaglutinine et marqués au chrome 51 et utilisés comme cellules cibles sont mis en présence du serum ou des lymphocytes du receveur. Quand l'allogreffe est constituée de corticale lyophilisée, l'immunisation n'est observée que chez l'un des six lapins. Par contre, lorsque l'allogreffe corticale ou médullaire est congelée, l'immunisation apparaît chez tous les animaux ; elle est cependant plus faible que celle observée avec des allogreffes fraîches.

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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The study of the immunogenicity of preserved bone allografts is of increasing interest because of the clinical use of these allografts. In the present work, a chromium release cytotoxicity test was used to study the cellular and/or humoral immunity of rabbits receiving freeze-dried and deep-frozen (-170°C) cortical or cancellous bone allografts. The allografts were placed orthotopically in the iliac crest of rabbits. The chromium-labeled (^{51}) lymphocytes of the donor stimulated with phytohemagglutinin were used as target

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cells in the presence of serum or the lymphocytes of the recipient. When the allograft consisted of freeze-dried cortical bone, immunization was noted only in one of the six rabbits. On the other hand, when the cortical or cancellous allograft was deep frozen, immunization appeared in all the animals, it was, however, lower than that observed with fresh allografts.